

# NG2 expressed by macrophages and oligodendrocyte precursor cells is dispensable in experimental autoimmune encephalomyelitis

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Increased expression of the chondroitin proteoglycan NG2 is a prominent feature in central nervous system injury with unknown cellular source and biological relevance. Here, we describe the first detailed analysis of experimental autoimmune encephalomyelitis in NG2 knockout mice and NG2 knockout bone marrow chimeras. We show that both macrophages and oligodendrocyte progenitor cells express and secrete NG2 in response to transforming growth factor- $\beta$ . A subpopulation of macrophages expresses NG2 within leucocyte infiltrates in the central nervous system, but only oligodendrocyte progenitor cells contribute to NG2 accumulation. Notably, NG2 plays no role in experimental autoimmune encephalomyelitis initiation, progression or recuperation. In concurrence, the immune response is unaltered in NG2-deficient mice as are the extent of central nervous system damage and degree of remyelination.

**Keywords:** multiple sclerosis; glial scar; macrophage; oligodendrocyte progenitor; TGF- $\beta$

**Abbreviations:** EAE = experimental autoimmune encephalomyelitis; TGF- $\beta$  = transforming growth factor- $\beta$

## Introduction

The glial scar is considered a major obstacle in effective recovery of the CNS from insult. Virtually all CNS injuries, including the autoimmune disease multiple sclerosis, trigger rapid activation of astrocytes, microglia, macrophages and oligodendrocyte progenitor cells (Fawcett and Asher, 1999; Alonso, 2005), which in turn deposit chondroitin sulphate proteoglycans (Dou and Levine, 1994;

Tang *et al.*, 2003; Laabs *et al.*, 2007). The high molecular weight chondroitin sulphate proteoglycan NG2 (CSPG-4) is a prominent component of the glial scar (Tang *et al.*, 2003; Tan *et al.*, 2005) and is considered a major obstacle for axon regeneration, remyelination and CNS recovery (de Castro *et al.*, 2005; Dou and Levine, 1994; Fawcett and Asher, 1999; Chen *et al.*, 2002; Morgenstern *et al.*, 2002; Larsen *et al.*, 2003; Tang *et al.*, 2003; Tan *et al.*, 2005, 2006; Laabs *et al.*, 2007), although this

view is now being questioned (Ughrin *et al.*, 2003; de Castro *et al.*, 2005; Tan *et al.*, 2006; Hossain-Ibrahim *et al.*, 2007).

Although the rapid accumulation of NG2 is a common feature of damage to the CNS, the biological role of NG2 in CNS injury is still a conundrum. The short intracellular domain of NG2 binds various signalling molecules that may link NG2 to synapses and the cytoskeleton. The large extracellular domain (290 kDa) contains several disulphide bonds, *N*-glycosylation sites and a single chondroitin chain, and binds a wide array of proteins including receptors, growth factors, extracellular matrix components and proteases, indicating that NG2 may regulate diverse cellular processes (Stallcup, 2002; Nishiyama *et al.*, 2009; Trotter *et al.*, 2010). Importantly, the extracellular domain of NG2 can be cleaved off and incorporated into the extracellular matrix (Nishiyama *et al.*, 1995; Asher *et al.*, 2005). The high concentration of proteases and large numbers of NG2 expressing cells at sites of inflammation in the CNS may explain the high amount of NG2 in the glial scar. The biological function of NG2 shedding in response to CNS injury remains to be addressed.

Surprisingly, the cellular source of NG2 in areas of CNS damage has become a matter of debate. Although oligodendrocyte progenitor cells are generally considered responsible for NG2 accumulation [NG2 is a marker for these cells (Nishiyama *et al.*, 2009; Trotter *et al.*, 2010)] recent reports indicate that activated microglia and CNS-infiltrating macrophages may also express NG2 after CNS injury (Bu *et al.*, 2001; Jones *et al.*, 2002; Yokoyama *et al.*, 2006; Fiedorowicz *et al.*, 2008; Matsumoto *et al.*, 2008; Smirkin *et al.*, 2009; Zhu *et al.*, 2010). Blood-derived monocytes and macrophages, and microglia play pivotal roles in CNS injury and can cause extensive CNS damage including axonopathy and demyelination, events that trigger substantial oligodendrocyte progenitor cell activation and glial scar formation (Di Bello *et al.*, 1999; Reynolds *et al.*, 2002; Alonso, 2005; Foote and Blakemore, 2005; Rhodes *et al.*, 2006; Mildner *et al.*, 2009). Importantly, NG2 may mediate the inflammatory response of activated microglia (Gao *et al.*, 2010). However, the signals triggering NG2 expression in macrophages and microglia are currently unknown and whether these cells do contribute to NG2 accumulation—and their large numbers at sites of inflammation and in the glial scar support such a notion—has not yet been addressed.

In this study, we have investigated the cellular source(s) of deposited NG2, the signals triggering NG2 expression and its function in myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis (EAE), the murine model system for multiple sclerosis. We show that transforming growth factor- $\beta$  (TGF- $\beta$ ) upregulates NG2 expression and secretion by oligodendrocyte progenitor cells and macrophages and demonstrate that NG2 is redundant in EAE progression, recruitment of leucocytes into the CNS, neuronal damage, demyelination and importantly, remyelination. Finally, we prove that blood-derived macrophages express NG2 in the CNS during EAE but do not contribute to the accumulation of NG2 at sites of inflammation. Our data excludes a prominent role for NG2 in immune regulation and regeneration in autoimmune inflammation of the CNS.

## Materials and methods

### Mice

Seven- to eight-week-old C57BL/6 female mice were purchased from Harlan. NG2 knockout mice were a kind gift from Prof. William B. Stallcup, Burnham Institute for Medical Research, and backcrossed to C57BL/6 background for nine generations.

To generate bone marrow chimeras, mice were lethally irradiated with a split-dose of 1000 rad. Donor animals were euthanized with CO<sub>2</sub> and bones (fore- and hind legs, hips) were flushed with sterile phosphate buffered saline to obtain bone marrow stem cells. In total,  $5 \times 10^6$  total bone marrow cells were injected intravenously per mouse. To prevent bacterial infection, 1% Neomycin (Sigma) was added for 2 weeks to the drinking water. After 6 weeks, prior to myelin oligodendrocyte glycoprotein immunization, reconstitution was verified by flow cytometry with anti-CD45.1 and anti-CD45.2 (BD PharMingen) on circulating leucocytes isolated from blood samples. Average reconstitution exceeded 97%.

All animal experiments were approved by the Swiss Veterinary Office (115/05; Zurich, Switzerland) and performed according to federal and institutional guidelines.

### Reagents

Cytokines, growth factors and chemicals were obtained from the following sources. Roche Diagnostics: mTNF- $\alpha$  and mIFN- $\gamma$ . PeproTech: rmIL-1b, hIL-2, mL-6, rmIL-13, rhTGF- $\beta$  2 and M-CSF. BD PharMingen: rmIL-10. Antibodies to NG2 were either a kind gift from Prof. William B. Stallcup or obtained from Chemicon. Anti- $\beta$ -tubulin-horseradish peroxidase and anti- $\beta$ -actin-horseradish peroxidase were purchased from Abcam and Sigma, respectively. Antibodies to CD3 (biotin), CD4 (PE), CD8 (FITC), CD11b (biotin), B220 (biotin) and Gr1 (FITC), were obtained from BD PharMingen and F4/80 (FITC) from Serotec. CD11b (unlabelled), CD16/32 (Fc block) and CD45 (APC) were purchased from Biolegend and CD11c (clone N418, PE) from Caltag. The secondary antibody anti-rabbit horseradish peroxidase was obtained from Pierce. Anti-rat Alexa 488 and anti-rabbit Alexa 594 were purchased from Molecular Probes/Invitrogen and Streptavidin-APC-Cy7 from Biolegend.

### Experimental autoimmune encephalomyelitis induction

Mice were injected with 100  $\mu$ g myelin oligodendrocyte glycoprotein<sub>35–55</sub> (Anawa) in 200  $\mu$ l phosphate-buffered saline: complete Freund's adjuvant (Difco) (1:1) subcutaneously on the right flank and with 300 ng Pertussis toxin (List Biological Laboratories) intraperitoneally (i.p.) on Day 0, followed by a boost of 300 ng Pertussis toxin at Day 2 and 100  $\mu$ g myelin oligodendrocyte glycoprotein<sub>35–55</sub> in complete Freund's adjuvant into the left flank on Day 7. The scoring of clinical symptoms was performed as described previously (Moransard *et al.*, 2010).

### Primary cells and cell lines

Primary oligodendrocyte progenitor cells were isolated as described previously (Moransard *et al.*, 2010). Briefly, neonatal forebrain cells were harvested from 1- to 2-day-old neonate C57/BL6 mice by dissociation of cortices in papain (Sigma) and grown in Dulbecco's

modified Eagle's medium (Gibco) supplemented with 10% horse serum (Gibco) on poly-D-lysine (Sigma)-coated cell culture flasks. After 10 days, the loosely attached oligodendrocyte progenitor cells were separated from the glial feeder layer by 16 h of mechanical shaking at 210 rpm, 37°C. The collected oligodendrocyte progenitor cells were further cultured either in Sato medium as described previously (Moransard *et al.*, 2010) or in Dulbecco's modified Eagle's medium/F12 (Gibco), 10 mM *N*-acetyl-L-alanyl-L-glutamine (L-GLU) (Biochrom AG), 1% Penicillin-Streptomycin (Gibco), 2% B-27 supplement (Gibco) on poly-D-lysine coated cell culture flasks.

Bone marrow-derived macrophages were prepared from bone marrow cells isolated from the femur and tibia. Bones were flushed with Hanks' balanced salt solution and the cell suspension was forced through a 70-µm mesh. Collected cells were resuspended in complete macrophage medium containing Dulbecco's modified Eagle's medium, 30% L929 cell-conditioned medium (source of macrophage colony-stimulating factor), 20% horse serum, 10 mM HEPES (Gibco) and 10 mM L-GLU and cultured at 37°C, 5% CO<sub>2</sub>. After 7 days, virtually 100% of the cells expressed the macrophage markers CD11b and F4/80. The cells were harvested by scraping on ice and frozen in 90% foetal calf serum and 10% dimethyl sulphoxide at a density of 5–10 × 10<sup>6</sup> cells/ml. Thawed cells were cultured in either Dulbecco's modified Eagle's medium containing 10% foetal calf serum (PAA Laboratories GmbH), 2 mM L-GLU and 20 µg/ml gentamicin (Sigma) or the same medium without serum.

BV2 cells (lab stock) were cultured in Dulbecco's modified Eagle's medium containing 10% foetal calf serum, 2 mM L-GLU and 20 µg/ml gentamicin.

OLneue cells (kindly provided by Dr J. Trotter) were cultured as described for primary oligodendrocyte progenitor cells.

To determine NG2 expression, all cell types were seeded on 3 cm culture dishes at 1.0–2.0 × 10<sup>6</sup> cells/dish and treated with cytokines for 48 h prior to real-time polymerase chain reaction or western blot analysis. For immunocytochemistry, cells were grown at similar densities on glass cover slips.

## Isolation of central nervous system mononuclear cells

CNS-mononuclear cells were isolated as described previously (Moransard *et al.*, 2010). Briefly, brains and spinal cords of animals perfused with Hank's balanced salt solution were minced with a scalpel blade and digested for 30 min at 37°C in Hank's balanced salt solution containing 50 µg/ml DNase I and 100 µg/ml collagenase/dispase (Roche). The digestion was quenched on ice and passed through a 100 µm nylon mesh (BD Biosciences) and centrifuged, after which the pellet was resuspended in 30% Percoll (Sigma). The gradient was centrifuged at 29 000 g for 30 min at 4°C (Kontron Instruments). The top layer containing myelin was removed by aspiration, and the interphase containing mononuclear cells was collected, diluted 3-fold with Hank's balanced salt solution and collected by centrifugation at 300 g.

## Flow cytometric analysis

Cells were resuspended in flowcytometry buffer containing 2% foetal calf serum, 5 mM ethylenediaminetetraacetic acid, 0.01% NaN<sub>3</sub> in phosphate buffered saline. Prior to staining with the appropriate antibodies, Fc receptors were blocked by incubation with anti-mouse CD16/32 (Fc-block, BD Pharmingen). 7-amino-actinomycin D (7-AAD) was used to exclude non-viable cells. All flow cytometric

data was obtained with a CyFlow flowcytometer (Partec). Data analysis was performed with FlowJo.

Infiltrating mononuclear cells were distinguished from CNS-resident microglia (CD45<sup>int</sup>/CD11b<sup>+</sup>/F4/80<sup>+</sup>) on the basis of their high CD45 expression. Within this population the following cell types were distinguished using gating as indicated between brackets: CD4 T cells (CD3<sup>+</sup>/CD4<sup>+</sup>), CD8 T cells (CD3<sup>+</sup>/CD8<sup>+</sup>), Granulocytes (CD11b<sup>+</sup>/Gr1<sup>+</sup>), lyDCs (lymphoid dendritic cells; CD11c<sup>+</sup>/CD11b<sup>+</sup>/F4/80<sup>+</sup>), myDCs (myeloid DCs; CD11c<sup>+</sup>/CD11b<sup>+</sup>/F4/80<sup>+</sup>), inflDCs (inflammatory DCs; CD11c<sup>+</sup>/CD11b<sup>+</sup>/F4/80<sup>+</sup>), pDCs (plasmacytoid DCs; CD11c<sup>+</sup>/B220<sup>+</sup>/Gr1<sup>+</sup>), B cells (CD11c<sup>+</sup>/B220<sup>+</sup>/Gr1<sup>+</sup>), Macrophages (CD11c<sup>+</sup>/CD11b<sup>+</sup>/F4/80<sup>+</sup>).

## Western blotting

To determine the amount of cell-bound NG2, cultured cells were scraped on ice in lysis buffer containing 1% Nonidet P40 (Igepal CA 630, Fluka), 50 mM Tris, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid and a cocktail of protease inhibitors (Complete Mini, Roche), after which the lysates were cleared from insoluble material by centrifugation. To determine the amount of secreted NG2, cell-conditioned media were collected and concentrated 10-fold using Amicon Ultra centrifugal filter devices with a cut-off of 100 kDa (Amicon). Both lysates and cell conditioned-media were incubated for 3 h at 37°C with Chondroitinase ABC (Sigma) in order to remove the chondroitin side chain of NG2 prior to incubation with an appropriate volume of Laemmli buffer at 80°C.

The protocol to isolate soluble and detergent-extractable NG2 fractions from CNS tissue is based upon the methods published by Asher *et al.* (2005). In brief, spinal cords isolated from EAE diseased mice were homogenized on ice with a douncer in the same buffer as used for cultured cells but omitting NP-40. The resulting lysate was cleared of insoluble material by low-speed centrifugation (3000 g, 10 min). The supernatant contains soluble NG2, whereas detergent-extractable NG2 was recovered by resuspending the pellet in lysis buffer containing 1% NP-40. Finally both lysates were cleared from remaining insoluble material by centrifugation (15 000 g, 10 min) followed by determination of the protein concentration using a BCA protein assay kit (Pierce) and incubation with an appropriate volume of Laemmli buffer at 80°C.

For western blotting for NG2 equal amounts of protein were loaded on NuPAGE 3–8% Tris-acetate gels (Invitrogen). Probing for β-tubulin and/or β-actin was performed to control for loading. Images of western blots were obtained and quantified using the GE Healthcare ImageQuant 350 system and accompanying software.

## Immunohistochemistry and immunocytochemistry

Mice were CO<sub>2</sub> anaesthetized and perfused with 25 ml Ringer solution (Braun Medical AG). Immunohistochemistry was performed on 10–15 µm frozen, 4% paraformaldehyde- or methanol-fixed longitudinal or cross sections of thoracic and lumbar spinal cord. Sections were thawed, fixed and blocked (5% foetal calf serum, 0.01% Triton, phosphate buffered saline) prior to incubation with primary antibody 1/200 in blocking buffer for 24 h. After washes with phosphate buffered saline, sections were incubated with the appropriate secondary antibody in block buffer for 2 h. After counterstaining with DAPI (Molecular Probes) sections were mounted in Mowiol embedding medium (Mowiol 4-88, Calbiochem) containing 0.1% 1,4-diazabicyclo-[2.2.2]-octan (DABCO, Fluka). Immunocytochemistry on cultured cells

was performed using the same protocol directly after a short wash and fixation of the cells with 4% paraformaldehyde for 10 min. Images were obtained on either a Zeiss Axiovert 40 or a Leica SP5 confocal microscope.

## Histology

Mice were sacrificed with CO<sub>2</sub>. Histology was performed as described recently (Prinz *et al.*, 2006). Spinal cords were removed and fixed in 4% buffered formalin and embedded in paraffin before staining with haematoxylin and eosin or Luxol fast blue to assess the degree of demyelination, MAC-3 (BD PharMingen) for macrophages and microglia, CD3 for T cells (Serotec), B220 (BD PharMingen) for B cells, APP for amyloid precursor protein (Chemicon) and glial fibrillary acidic protein for astrocytes (Dako).

## Electron microscopy and evaluation of remyelination

Electron microscopy and evaluation of remyelination was performed as recently described (Raasch *et al.*, 2011). Epon-embedded, glutaraldehyde-fixed spinal cord slices from lumbar spinal cords (L1–L4) from five wild-type and five NG2 animals at Day 40 post-immunization. Four wild-type and three NG2 age- and sex-matched healthy control animals were prepared, cut and stained with toluidine blue. This staining allowed clear distinction between normal and inflamed white matter by light microscopy. The tissue was then trimmed and reoriented so that ultrathin cross sections of the spinal cord could be cut and treated with uranyl acetate and lead citrate. Thirty electron micrographs from within spinal cord sections were obtained at  $\times 13\,500$  magnification from each animal. Of these, 10 images for each animal were randomly selected and axon diameter and myelin thickness were measured using the analySIS Dock System (Soft Imaging System GmbH). Within each image all axons with diameters of  $>250$  nm were evaluated, axons with smaller diameters were omitted, since these are usually unmyelinated axons. This meant that at least 130 axons per animal were evaluated. G-ratios were defined as diameter of the axon divided by fibre diameter (axon plus myelin). We calculated a G-ratio for each fibre and subsequently averaged all G-ratios from one spinal cord. In addition, the number of myelinated fibres in relation to all fibres was expressed as percentage of myelinated axons to all visible axons. Electron micrographs were obtained using a Philips CM 100 electron microscope.

## RNA isolation and real-time polymerase chain reaction

Whole-cell RNA from cultured cells was extracted using the NucleoSpin-RNA II kit (Macherey–Nagel). RNA from mouse tissues was extracted by homogenization in TRIzol<sup>®</sup> (Invitrogen) according to the manufacturer's instructions. RNA was reverse-transcribed using random hexamers and AMV reverse transcriptase (Promega). The complementary DNA equivalent to 50 ng of total RNA was amplified by polymerase chain reaction in an ABI PRISM 7700 detection system (PE–Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems) and quantified using the  $2^{-\Delta\Delta CT}$  method using 18S ribosomal RNA as a housekeeping gene. Relative RNA levels are expressed as fold change compared to control. Primers and probes for Taqman analysis for IL-2, IL-6, IL-10, TNF- $\alpha$ , IFN- $\gamma$ , TGF- $\beta$  and NG2 were purchased from Applied Biosystems.

## Proliferation assay

OL1neu were seeded in a 96-well plate and treated with cytokines for 16 h before the addition of  $5\mu\text{Ci/ml}$  <sup>3</sup>H-thymidine (GE Healthcare). After incubation for 16 h at 37°C the cells were harvested directly onto a glass fibre filter membrane using a 96-well plate harvester. The filters were dried at 60°C, sealed in a plastic bag containing scintillation fluid (PerkinElmer) and measured in a beta-counter (PerkinElmer).

## Statistical analysis

For data obtained *in vivo* we performed unpaired, two-tailed Student's *t*-tests assuming equal variances. For data obtained *in vitro* we performed paired, two-tailed Student's *t*-tests assuming equal variances. Numbers following the  $\pm$  sign represent standard error (SEM).

## Results

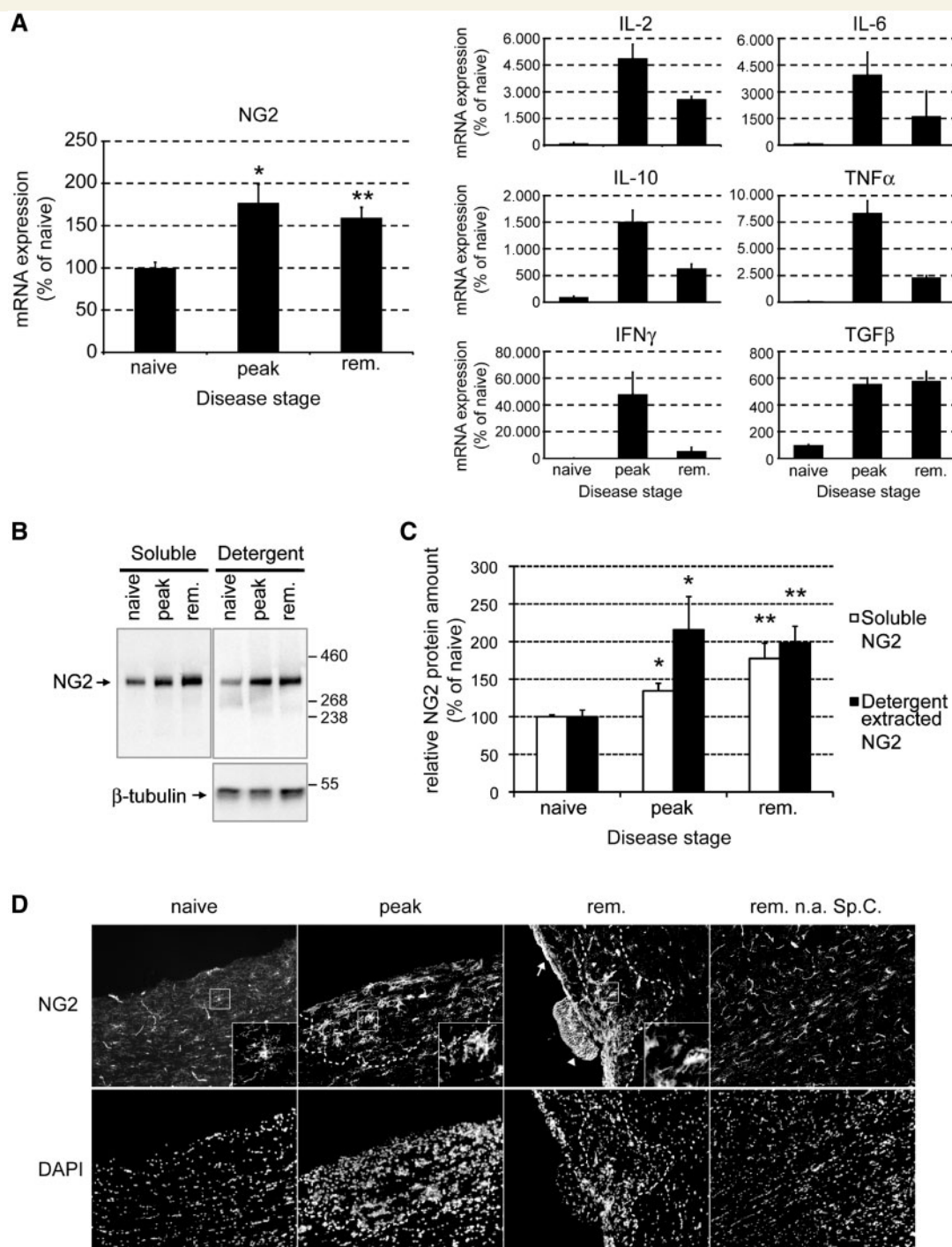
### NG2 protein accumulates at sites of infiltrating leucocytes in experimental autoimmune encephalomyelitis

In the injured CNS, NG2 protein levels are often elevated at sites of tissue damage and/or inflammation and increased NG2 levels remain associated with the glial scar (Levine, 1994; Fawcett and Asher, 1999; Morgenstern *et al.*, 2002; Alonso, 2005; Tan *et al.*, 2005, 2006). However, in EAE and multiple sclerosis, changes in NG2 levels have been less well described (Di Bello *et al.*, 1999; Reynolds *et al.*, 2002). Examination of NG2 and cytokine messenger RNA expression by quantitative real-time polymerase chain reaction in the spinal cord at different EAE disease stages showed that at peak disease on Day 18 post-immunization (average score  $2.78 \pm 0.15$ ) NG2 messenger RNA levels are significantly elevated and remain elevated in the remission phase (Day 43 post-immunization, average score  $2.13 \pm 0.07$ ) (Fig. 1A). Notably, the NG2 expression profile correlates well with the expression of TGF- $\beta$  in the spinal cord, with high expression maintained in the remission phase (Fig. 1A). In contrast messenger RNA expression levels of IL-2, IL-6, IL-10, TNF- $\alpha$  and IFN- $\gamma$  subsided after the peak phase (Fig. 1A).

Since NG2 exists in both cell-bound and secreted forms (Nishiyama *et al.*, 1995; Stallcup, 2002; Asher *et al.*, 2005), we examined the expression of both soluble and detergent-extracted NG2 protein in spinal cord at different EAE stages by western blot (Fig. 1B). Both the 300 kDa cell-bound and 290 kDa secreted forms of NG2 are significantly elevated during EAE. The highest amount of detergent-extracted NG2 is detected at the peak phase whereas soluble NG2 increased over the course of the disease (Fig. 1B and C).

We investigated by immunohistochemistry if the increase in NG2 protein was restricted to sites of leucocyte infiltration in the spinal cord (Fig. 1D). In the spinal cord of healthy mice (naïve), NG2 is expressed by pericytes associated with blood vessels, meningeal cells and oligodendrocyte progenitor cells (insert shows the typical stellar appearance of oligodendrocyte progenitor cells).





**Figure 1** NG2 is upregulated and accumulated in and around areas of leucocyte infiltration at peak and remitting stages of EAE. (A) Quantitative real-time polymerase chain reaction analysis of NG2 and cytokine messenger RNA expression in spinal cord at different EAE stages. NG2 messenger RNA expression is significantly increased at peak and remission (rem.). The expression of NG2 messenger RNA correlates well with TGF- $\beta$  messenger RNA expression, but not with the expression of IL-2, IL-6, IL-10, TNF- $\alpha$  or IFN- $\gamma$ . (B and C) Western blot and corresponding densitometric quantitation of soluble and detergent-extracted NG2 in spinal cord lysates from EAE diseased mice. Both soluble and detergent-extracted NG2 protein amounts are significantly elevated in spinal cord at peak and remission. (D) Immunohistochemistry for NG2 in longitudinal sections of spinal cord. In the spinal cord of naïve animals NG2 expression is confined to blood vessels and oligodendrocyte progenitor cells. Insert shows an electronically  $\times 3$  magnified image of a stellate oligodendrocyte progenitor cell in the boxed area. NG2 expression typical for the meninges (arrow) and satellite cells of the dorsal root ganglia (arrow head) are illustrated in the remission panel. At peak and remission, NG2 immunoreactivity is increased in and around areas of infiltrating leucocytes, identified by DAPI staining as clustered nuclei. NG2 immunoreactivity is diffuse and does not resemble the classic oligodendrocyte progenitor cell morphology. Inserts show electronically  $\times 3$ -magnified images of the boxed areas. In normal appearing spinal cord at remission (rem. n.a. Sp.C.), NG2 immunoreactivity is not increased and is localized to oligodendrocyte progenitor cells and blood vessels. Bars represent mean  $\pm$  SEM;  $n = 4$ ; \* $P < 0.05$ ; \*\* $P < 0.01$  versus naïve wild-type control.

NG2 expression typical for the meninges (arrow) and satellite cells of the dorsal root ganglia (arrow head) are illustrated in the remission panel. At peak disease, NG2-immunoreactivity is clearly elevated within and around the borders of the area occupied by infiltrating leucocytes (outlined area). Importantly, within and in close proximity of these sites of leucocyte infiltration, Olig2<sup>+</sup> oligodendrocyte progenitor cells are abundantly present (Supplementary Fig. 3). Note that within infiltrates some areas are completely devoid of NG2-immunoreactivity, probably a sign of extensive tissue damage. The insert illustrates that NG2 is no longer restricted to stellar oligodendrocyte progenitor cells but now has a rather diffuse distribution, which is maintained at the remission stage of EAE. In contrast, in the normal appearing spinal cord at remission, distribution of NG2 is indistinguishable from its distribution in healthy mice. Thus both cell-bound and secreted NG2 are upregulated in the spinal cord during EAE and accumulate at sites of leucocyte infiltration.

## TGF- $\beta$ increases NG2 expression and secretion by macrophages and oligodendrocyte progenitor cells

During EAE the CNS is subjected to a steep increase in the concentration of disease-mediating cytokines (Fig. 1A) that could potentially trigger NG2 accumulation by increasing oligodendrocyte progenitor cell numbers and/or NG2 expression by oligodendrocyte progenitor cells. In addition to oligodendrocyte progenitor cells, both macrophages and the CNS-resident microglia are potential producers of NG2 (Bu *et al.*, 2001; Jones *et al.*, 2002; Yokoyama *et al.*, 2006; Fiedorowicz *et al.*, 2008; Matsumoto *et al.*, 2008; Smirkin *et al.*, 2009; Zhu *et al.*, 2010).

We treated bone marrow-derived macrophages and the oligodendrocyte progenitor cell line OLIneu with a variety of cytokines with known roles in EAE, followed by quantitative real-time polymerase chain reaction analysis. Notably, only TGF- $\beta$  increases expression of NG2 messenger RNA in both OLIneu and macrophages (Fig. 2A). In addition IL-6 increased NG2 expression in OLIneu to a similar extent. Subsequent <sup>3</sup>H-thymidine incorporation assays indicated that the TGF- $\beta$ -induced increase in NG2 messenger RNA in OLIneu is not due to enhanced proliferation (Supplementary Fig. 1). To test if primary oligodendrocyte progenitor cells are capable of secreting NG2 and if secreted and/or cell-bound NG2 protein levels are altered by TGF- $\beta$ , oligodendrocyte progenitor cell-conditioned media and cell lysates were analysed by western blotting (Fig. 2B). TGF- $\beta$  treatment of primary oligodendrocyte progenitor cells demonstrated that both secreted and cell-bound NG2 amounts increase after 48 h. A time course of TGF- $\beta$  treatment of OLIneu confirmed these results (Supplementary Fig. 2). Collectively these data show that TGF- $\beta$  treatment of oligodendrocyte progenitor cells induces an increase in cell-bound and secreted NG2 without influencing cell proliferation.

To investigate if macrophages and/or microglia express NG2 protein and possibly secrete NG2 in response to cytokines we treated bone-marrow derived macrophages and the microglial cell line BV2 (Bocchini *et al.*, 1992) with IL-10, TNF- $\alpha$ , IFN- $\gamma$  or

TGF- $\beta$  and analysed their conditioned media and cell lysates by western blotting. Although untreated macrophages express a small amount of NG2, TGF- $\beta$  triggers a significant increase in both cell-bound and secreted NG2 (Fig. 2C and D). In contrast, untreated microglia do not express NG2, and TGF- $\beta$  induced expression of a small amount of cell-bound, but not secreted, NG2 (Fig. 2E). Notably, a comparative western blot showed that the amount of cell-bound NG2 expressed by microglia is negligible and barely visible compared to the amount produced by either macrophages or oligodendrocyte progenitor cells, with the latter producing the highest amount of cell-bound NG2 (Fig. 2F).

Subsequent immunocytochemical analysis (Fig. 2G) showed that OLIneu cells are always NG2<sup>+</sup> and TGF- $\beta$  treatment elicits a modest increase in NG2 immunoreactivity. Untreated macrophages are primarily NG2<sup>−</sup> although occasionally a NG2<sup>+</sup> cell is detected. TGF- $\beta$  treatment triggers a substantial increase in the number of NG2<sup>+</sup> macrophages but the intensity of NG2-immunoreactivity varies between cells. Notably, NG2 immunoreactivity was never observed in microglia after any of the treatments. In summary, these data indicate that both macrophages and oligodendrocyte progenitor cells upregulate NG2 expression and secretion in response to TGF- $\beta$ , whereas microglia only produce cell-bound NG2 at low levels in response to TGF- $\beta$  and do not secrete NG2.

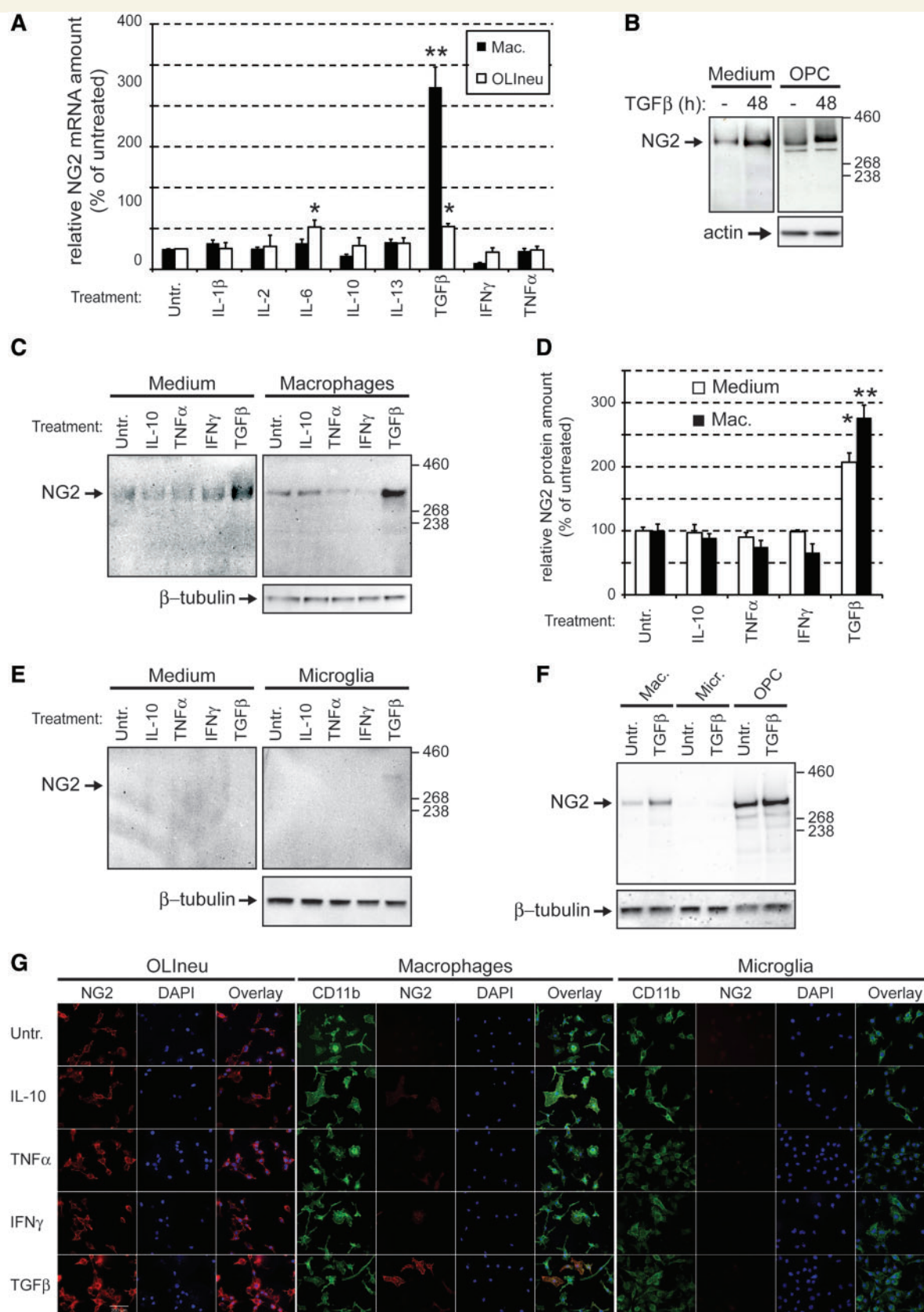
## NG2 is not required in experimental autoimmune encephalomyelitis

The TGF- $\beta$ -induced increase in NG2 expression in oligodendrocyte progenitor cells and macrophages, which may underlie the accumulation of NG2 at sites of leucocyte infiltration, is likely to be an important response of the CNS to injury; however, its function is currently unclear.

Therefore, we subjected NG2-deficient (NG2 knockout) mice (Grako *et al.*, 1999; de Castro *et al.*, 2005; Kucharova and Stallcup, 2010) and littermate control (wild-type) mice to EAE and followed disease progression (Fig. 3A) and weight loss (Fig. 3B). The absence of NG2 has no significant effect on EAE onset, disease severity or the extent or rate of recovery.

To investigate if the lack of NG2 influences demyelination, neuronal damage and/or astrocyte activation, histology on thoracic spinal cord cross-sections was performed (remission stage) (Fig. 3C). Quantitation of Luxol fast blue staining revealed that the extent of demyelination is similar in NG2 knockout and wild-type mice (Fig. 3D). Also the number of APP<sup>+</sup> deposits, indicative of damaged neurons, and glial fibrillary acidic protein<sup>+</sup> astrocytes are comparable in wild-type and NG2 knockout animals.

The expression of NG2 by macrophages *in vitro* and the accumulation of NG2 at sites of leucocyte infiltration suggested that NG2 might modulate the inflammatory response in EAE. Therefore different leucocyte subsets were monitored during the course of EAE in the CNS, spleen and inguinal lymph nodes by histology and flow cytometry but were not found to differ between NG2 knockout and wild-type mice (Fig. 3C–E; spleen and lymph node data not shown). Specifically, histology showed that at remission (Day 29) infiltrating T-cells (CD3), B-cells (B220) and macrophages



**Figure 2** TGF-β induces expression and secretion of NG2 by macrophages and oligodendrocyte progenitor cells *in vitro*. (A) Analysis of NG2 messenger RNA expression in (primary bone marrow-derived) macrophages (Mac.) and OLIneu treated with different cytokines (24 h) by quantitative real-time polymerase chain reaction. NG2 expression is significantly increased by treatment with TGF-β in OLIneu and macrophages. IL-6 also increases NG2 expression in OLIneu. (B) Representative western blot for NG2 of primary oligodendrocyte

(continued)



(Mac-3) are present in comparable numbers in the CNS of wild-type and NG2 knockout mice (Fig. 3C and D). In agreement, flow cytometric analysis at remission did not reveal differences in any of the leucocyte populations tested, including T cells, granulocytes, different dendritic cell types, microglia, and importantly, blood-derived macrophages (Fig. 3E, CNS).

Since NG2 is prominently accumulated within and around areas of leucocyte infiltration, we assessed whether the size and location of the infiltrates within the thoracic and lumbar spinal cord (lumbar not shown) are altered in NG2 knockout mice during the peak phase by immunohistochemistry (Fig. 3F). Investigation of the distribution of macrophages/activated microglia (CD11b and F4/80) indicates that cell number, total area covered by the cells and penetration of the cells into the spinal cord in relation to the meninges are comparable in wild-type and NG2 knockout mice. Note though the quite prominent overlap in macrophage distribution (CD11b and F4/80) and NG2 immunoreactivity in wild-type mice.

Finally, because remyelination may be inhibited by NG2 accumulation (Larsen *et al.*, 2003) or delayed by NG2-deficiency (Kucharova and Stallcup, 2010), we determined the extent and quality of remyelination by electron microscopy at Day 40 post-immunization and in healthy control animals of each genotype (Fig. 4A–E). Quantitation of the percentage of myelinated axons, axon diameter, myelin thickness and G-ratio indicates that axon integrity and remyelination are comparable between healthy wild-type and healthy NG2 knockout mice and at Day 40 post-immunization between both genotypes. As expected though, myelination is reduced in Day 40 post-immunization animals of both genotypes compared to myelination in healthy controls. The similar increase in the G-ratio at Day 40 post-immunization strongly suggests that initial demyelination was extensive in the experimental groups. The comparable G-ratios between wild-type and NG2 knockout animals at Day 40 post-immunization, however, show that remyelination occurs with similar efficiency in both genotypes. In summary, NG2 deficiency has no influence on EAE disease outcome, the extent of CNS tissue damage, cellular inflammatory response, demyelination or remyelination.

## Central nervous system resident cells are responsible for NG2 accumulation within and around infiltrates

Although our *in vitro* data indicates that both oligodendrocyte progenitor cells and macrophages upregulate and secrete NG2 in

response to TGF- $\beta$ , it still remains to be established if these cell types actually contribute to NG2 accumulation at sites of leucocyte infiltration in the CNS during EAE. Furthermore, it is possible that NG2 expression by oligodendrocyte progenitor cells or macrophages may have different or even opposite functions in CNS inflammation. To resolve these issues the following bone marrow chimeras were created: wild-type mice containing NG2 knockout bone marrow (knockout  $\rightarrow$  wild-type) resulting in mice with NG2 deficiency only in the immune compartment, NG2 knockout mice containing wild-type bone marrow (wild-type  $\rightarrow$  knockout) creating mice that express NG2 only in bone marrow-derived leucocytes, and wild type mice containing wild-type bone marrow (wild-type  $\rightarrow$  wild-type) as controls. We subjected these different groups to EAE and, in good agreement with the previous results (Fig. 3), weight-loss, onset, severity and progression of EAE were all comparable in the different chimeras (Fig. 5A and B) indicating that CNS- and leucocyte-derived NG2 do not have opposing or compensatory roles.

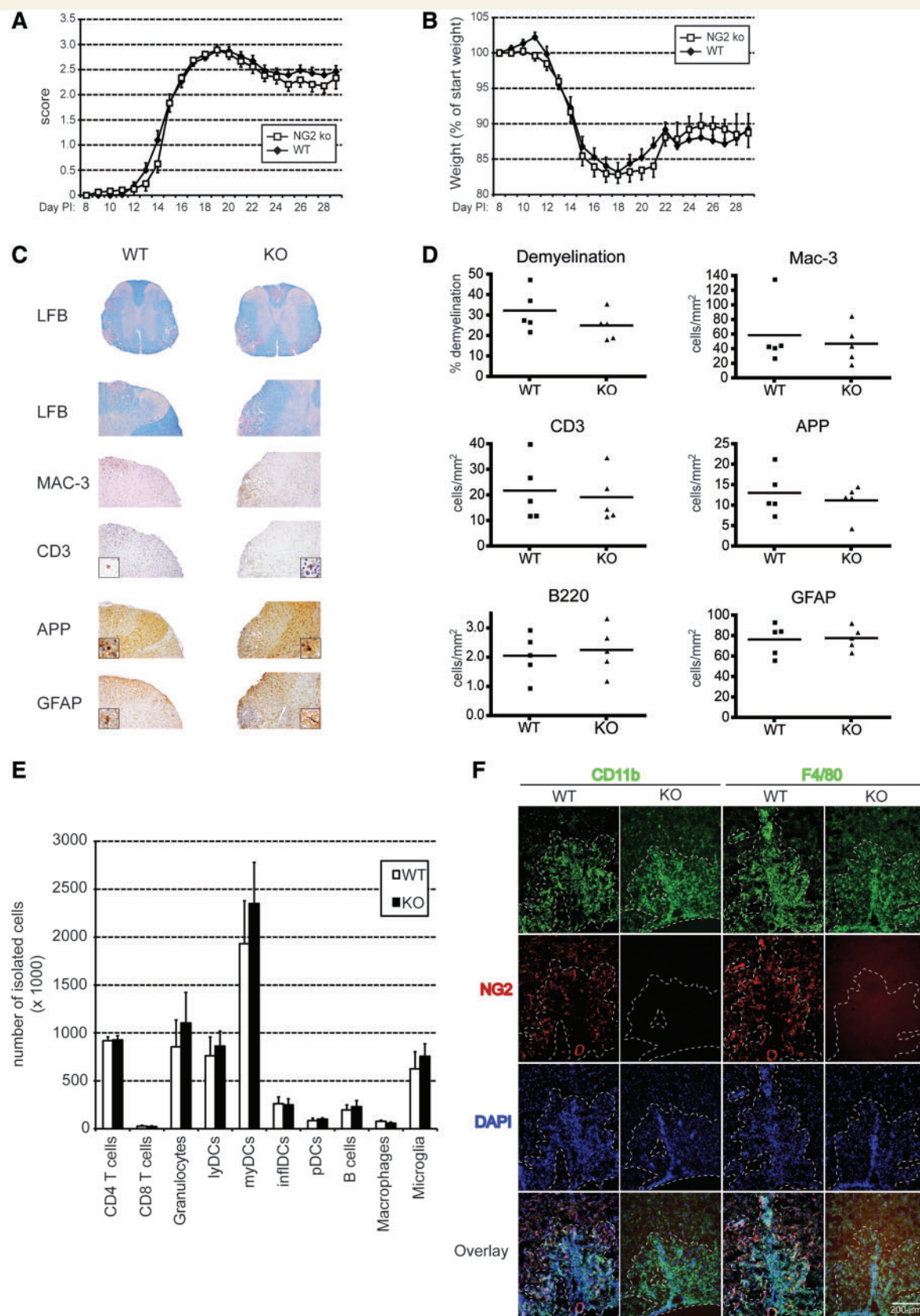
To determine the relative contribution of CNS-resident and CNS-infiltrating cells to the increase in NG2 in the spinal cord during EAE, the expression of soluble and detergent-extracted NG2 was examined by western blot (remission) (Fig. 5C and D). Both 300-kDa cell-bound and 290-kDa shed NG2 are significantly elevated in wild-type  $\rightarrow$  wild-type and knockout  $\rightarrow$  wild-type chimeras compared to NG2 levels in naïve wild-type mice. Notably, the amounts of soluble and cell-bound NG2 do not differ significantly between these two chimeras. In sharp contrast, in spinal cord lysates from wild-type  $\rightarrow$  knockout chimeras cell-bound and soluble NG2 were never detected by western blotting.

Analysis of thoracic spinal cord for NG2 and CD11b immunoreactivity by confocal microscopy showed that the location and number of infiltrating CD11b<sup>+</sup> leucocytes do not vary significantly between the different chimeras (Fig. 5D). However, the distribution of NG2 is strikingly different. Wild-type  $\rightarrow$  wild-type chimeras show NG2 accumulated within and around the area covered by the infiltrating cells and co-localization of CD11b and NG2-immunoreactivity can on occasion be observed (inserts). Importantly, the distribution and accumulation of NG2 in the knockout  $\rightarrow$  wild-type chimeras is comparable and also in these chimeras occasional CD11b and NG2 co-localization can be seen, albeit significantly less frequently. In contrast, increased NG2 expression and accumulation at sites of leucocyte infiltration are clearly abolished in wild-type  $\rightarrow$  knockout chimeras. Within the infiltrate occasionally NG2-expressing cells (insert), which are also

### Figure 2 Continued

progenitor cell lysates and culture medium after 48 h of TGF- $\beta$  treatment. TGF- $\beta$  upregulates both cell bound and secreted NG2. (C and D) Western blot for NG2 of macrophage lysates and culture medium after cytokine treatment (24 h) and corresponding densitometric quantitation. Untreated macrophages (Untr.) express and secrete a small amount of NG2. TGF- $\beta$  elicited a significant increase in cell-bound and secreted NG2. (E) Treatment of the microglia cell line BV2 with cytokines followed by western blot analysis of cell lysates and culture media. Microglia express cell-bound NG2 only after TGF- $\beta$  treatment. Secreted NG2 could not be detected. (F) Comparative western blot of NG2 expression in macrophages, microglia (Micr.) and primary oligodendrocyte progenitor cells (OPCs). Macrophages express a modest amount of NG2 although at lower levels than expressed by oligodendrocyte progenitor cells. NG2 expressed by microglia is barely detectable. (G) Immunocytochemistry for NG2 in OL1neu, macrophages and microglia after cytokine treatment. TGF- $\beta$  treatment induces an increase in NG2 immunoreactivity in OL1neu. TGF- $\beta$  elicits NG2 expression in most, but not all, macrophages (co-stained with CD11b). NG2 immunoreactivity is never detected in microglia. Bars represent mean  $\pm$  SEM;  $n = 4$ ; \* $P < 0.05$ ; \*\* $P < 0.01$  versus control.





**Figure 3** NG2 deficiency does not alter EAE disease progression or inflammatory profile. (A) EAE progression monitored in mice immunized with myelin oligodendrocyte glycoprotein using a method that scores the degree of paralysis on a scale of 1–5. Shown are the combined results of two independent experiments with 24 NG2 deficient (NG2ko) and 36 wild-type (WT) animals in total. (B) Progression of weight loss of the same mice. The fluctuations in weight correlate well with the degree of disability. NG2-deficiency has no effect on

(continued)

dimly CD11b<sup>+</sup>, can be observed. NG2-expressing cells were never detected outside infiltrates in wild-type → knockout chimeras. Notably though, the majority of CD11b<sup>+</sup> leucocytes do not express NG2. On average, 3–4 NG2<sup>+</sup> cells are present within an infiltrate, which are all faintly CD11b<sup>+</sup>.

Taken together, these results indicate that, although some blood-borne NG2<sup>+</sup>/CD11b<sup>+</sup> macrophages are present in the CNS during EAE, CNS resident cells are responsible for the increase in NG2 and its accumulation at sites of leucocyte infiltration.

## Discussion

### Oligodendrocyte progenitor cells are the source of accumulated NG2 at sites of leucocyte infiltration

The prominent increase in NG2 messenger RNA and protein in the CNS during the course of EAE can in theory be derived from various cellular sources. In particular oligodendrocyte progenitor cells, CNS-infiltrating macrophages and CNS-resident microglia are likely to express or upregulate NG2 during EAE. Indeed, our *in vitro* data show that all three cell types are able to express or upregulate NG2 expression in response to TGF- $\beta$  but not in response to various other cytokines that have been shown to play a role in EAE. In addition, of the various cytokine expression profiles we tested, only expression of TGF- $\beta$  correlated well with the expression of NG2 over the course of EAE, suggesting that TGF- $\beta$  signalling may trigger increased NG2 expression in EAE.

To determine which of the aforementioned cell types contribute to NG2 accumulation in the CNS, we induced EAE in various NG2 knockout bone marrow chimeras. Our data show that large amounts of NG2 are only accumulated in and around areas of leucocyte infiltration if the recipient mice have a wild-type background (knockout → wild-type and wild-type → wild-type chimeras) showing that increases in NG2 are derived from a CNS-resident cell type. Although it is possible that the lack of NG2 on peripheral cells, in particular leucocytes, may hamper CNS infiltration, which in turn could explain the lack of NG2 accumulation in the CNS, our immunohistochemical and flow cytometric data (latter not shown for chimeras) showing a comparable

degree of cellular infiltration and lesion size argues against this possibility.

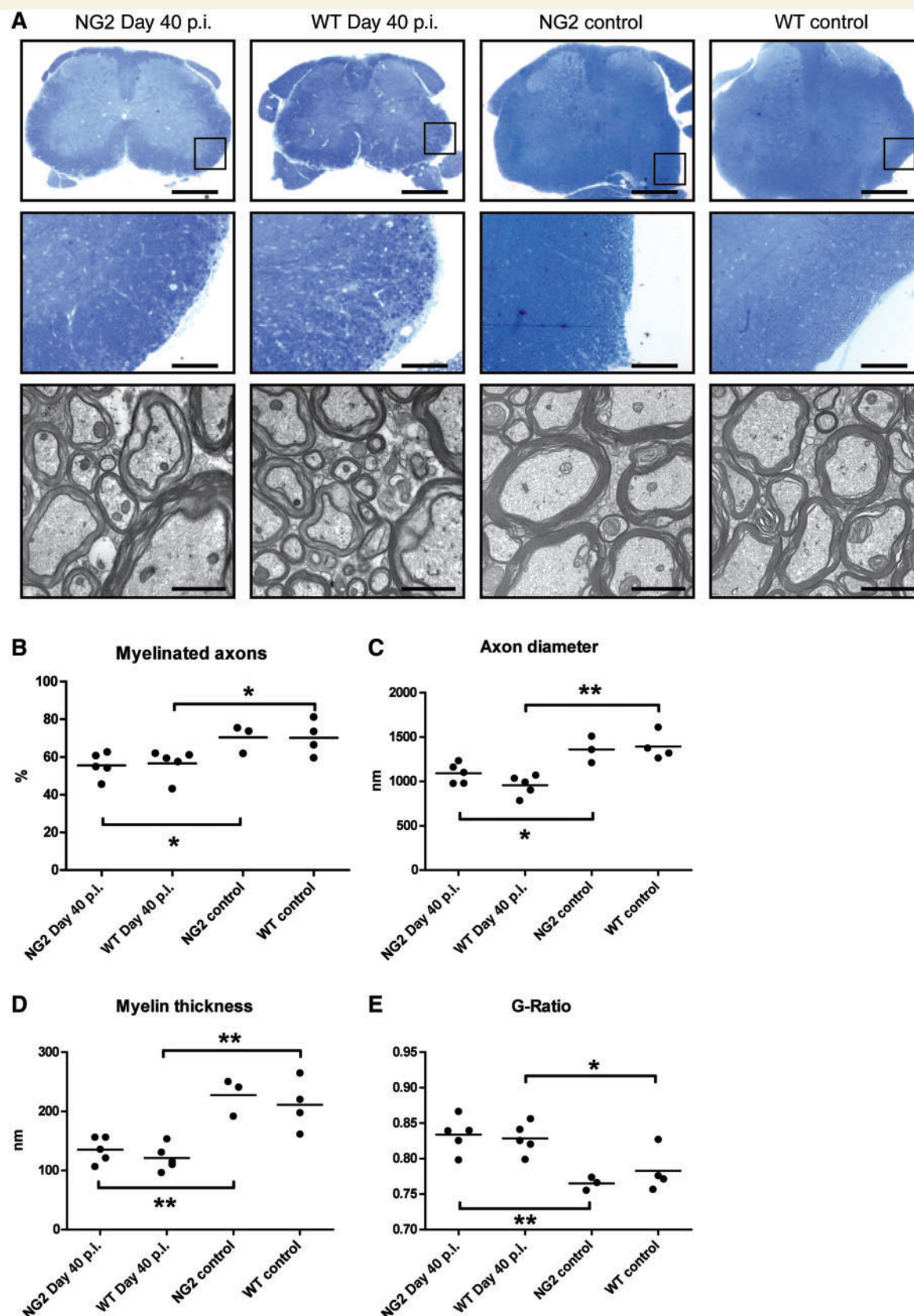
Notably, in wild-type → knockout chimeras, in which NG2 is expressed by bone marrow-derived cells (leucocytes) and not by CNS resident cells, only a small amount of NG2 is present in the CNS at peak EAE. Notably, lesion size and leucocyte infiltration into the CNS are comparable to control EAE-diseased mice. This small amount of NG2 is expressed solely by not yet fully characterized, but bone marrow-derived, CD11b<sup>+</sup> amoeboid-shaped cells. The actual amount of NG2 expressed by these cells in the spinal cord of wild-type → knockout chimeras was too low to be detected by western blot. Thus, NG2 found in and around lesion sites in the CNS during the course of EAE is derived from CNS resident cells rather than from leucocytes infiltrating from the periphery into the CNS, practically ruling out blood-borne macrophages as a major source of NG2 in EAE. These data agree with observations by Rhodes *et al.* (2006) showing that NG2 increases before ED1<sup>+</sup> macrophages infiltrate into lipopolysaccharide-injected rat brains.

Arguably, microglia as one of the potential brain resident sources of NG2, are unlikely to contribute to NG2 accumulation during EAE because of two observations. Firstly, even in the presence of TGF- $\beta$ , the amount of cell-bound NG2 expressed by microglia *in vitro* is negligible, and secreted NG2 is not produced at all by microglia, which is in good agreement with microarray data from our lab indicating that NG2 is not expressed by TGF- $\beta$ -treated primary microglia (Paglinawan *et al.*, 2003). Secondly, in knockout → wild-type chimeric mice, in which NG2 is only expressed by CNS resident cells and not by leucocytes, only sporadic co-localization between NG2 and the microglial/macrophage marker CD11b is observed despite leucocyte infiltration into the CNS and lesion sizes being indistinguishable from control EAE-diseased mice. Although the latter observation suggests that some microglia may express NG2 during EAE, the amount produced by these cells cannot account for the large NG2 deposits observed in and around infiltrates.

Taken together, our data suggest that oligodendrocyte progenitor cells responding to TGF- $\beta$  are the source of accumulated NG2 in areas of CNS injury because Olig2<sup>+</sup> oligodendrocyte progenitor cells are prominently present in and around infiltrates (Supplementary Fig. 3) and, *in vitro*, oligodendrocyte progenitor cells produce significantly larger amounts of both cell-bound and soluble NG2 in response to TGF- $\beta$  than macrophages and microglia. Our data is in accord with the observation that a single

#### Figure 3 Continued

EAE progression. (C and D) Histology and corresponding quantitation of thoracic spinal cord cross sections from EAE diseased NG2 knockout and wild-type mice at the remission stage for myelin loss (LFB = Luxol fast blue), neuronal damage (APP = amyloid precursor protein), astrocyte proliferation (GFAP = glial fibrillary acidic protein), and infiltration of macrophages (MAC-3) and T-cells (CD3). In addition the number of infiltrating B cells was assessed (B220). No significant differences in the number of infiltrating cells or degree of demyelination or neuronal damage were detected ( $n = 5$ ). (E) Flow cytometric analysis of CNS-infiltrating leucocytes. No significant differences were found in any of the leucocyte populations tested. For gating strategy and definition of leucocyte populations refer to the 'Methods' section. Bars represent mean  $\pm$  SEM;  $n = 4$ . (F) Immunohistochemistry for macrophages/microglia (CD11b and F4/80) on thoracic spinal cord cross-sections (peak). In wild-type animals, NG2 accumulation appears associated with infiltrates of CD11b and F4/80 positive macrophages/microglia. The number of infiltrating cells and area occupied does not noticeably differ between wild-type and NG2 knockout animals (scale bar = 200  $\mu$ m). PI = post-immunization.



**Figure 4** Normal myelination and remyelination in the absence of NG2. (A) Spinal cord sections from wild-type and NG2 animals Day 40 post-immunization (p.i.) and from sex- and age-matched healthy controls. (Top) Toluidine blue stained sections (scale bars = 500  $\mu$ m), box

(continued)



injection of TGF- $\beta$  into the rat basal ganglia elicits oligodendrocyte progenitor cell activation and increased NG2 immunoreactivity (Rhodes *et al.*, 2006). Functionally, TGF- $\beta$  may induce a reactive phenotype in oligodendrocyte progenitor cells with a concomitant increase in NG2 expression, which may precede differentiation into oligodendrocytes and subsequent remyelination (McKinnon *et al.*, 1993).

Notably, the localization of accumulated NG2 at sites of leucocyte infiltration appears rather diffuse and has a lack of distinct cellular structure indicating that NG2 is partly secreted and incorporated into the extracellular matrix, which is confirmed by our western blot data showing significant increases in both saline-soluble and detergent-extracted NG2. Interestingly, the shedding of NG2 appears to be a common response in CNS injury because increases in both secreted and cell-bound NG2 have also been described in knife lesions of rat cerebral cortex (Asher *et al.*, 2005). Although the functional significance of secreted NG2 is currently unclear, the ability of the shed domain to bind various growth factors and extracellular matrix components suggests that it may function as a molecular 'sink', sequestering growth factors and thereby limiting cell migration and/or proliferation. However, our data clearly show that NG2 does not limit leucocyte proliferation and migration within the CNS or impair clinical recovery in EAE. Notably, the latter observation, the presence of Olig2<sup>+</sup> oligodendrocyte progenitor cells in and around areas of leucocyte infiltration (Supplementary Fig. 3), and the comparable extent of remyelination between NG2 knockout and wild-type mice at the late remission phase also imply that oligodendrocyte progenitor cell migration, proliferation and differentiation are not majorly hampered by either NG2 accumulation or by NG2 deficiency.

Unfortunately, the diffuse appearance of the NG2 staining in infiltrates also makes it impossible to determine the number of NG2<sup>+</sup> oligodendrocyte progenitor cells and NG2<sup>+</sup>/CD11b<sup>+</sup> macrophages within these infiltrates by immunohistochemistry. Additional attempts to quantify these cell populations by flow cytometric analysis were also unsuccessful due to the lack of anti-NG2 antibodies that work in flowcytometry.

## NG2<sup>+</sup> macrophages in experimental autoimmune encephalomyelitis

Many recent reports have suggested that macrophage- and/or microglia-like NG2<sup>+</sup> cells accumulate in the CNS after a variety of insults (Bu *et al.*, 2001; Jones *et al.*, 2002; Hampton *et al.*, 2004; Rezajooi *et al.*, 2004; Yokoyama *et al.*, 2006; Fiedorowicz *et al.*, 2008; Matsumoto *et al.*, 2008; Smirkin *et al.*, 2009; Gao *et al.*, 2010; Wu *et al.*, 2010; Zhu *et al.*, 2010). Despite this large

body of data, claims made in earlier studies indicating that NG2<sup>+</sup> cells are quite distinct from macrophages and microglia still cast some doubts on the existence of NG2<sup>+</sup> macrophages (Levine, 1994; Nishiyama *et al.*, 1997; Di Bello *et al.*, 1999; Dawson *et al.*, 2000). Some of these original studies have argued that the occasional NG2<sup>+</sup> microglia/macrophage engulfed or phagocytosed rather than synthesized NG2 (Levine, 1994; Nishiyama *et al.*, 1997). Our data show that the existence of a macrophage-like NG2<sup>+</sup> cell in the CNS after insult can no longer be logically disputed. NG2<sup>+</sup>/CD11b<sup>+</sup> cells are clearly present in leucocyte infiltrates in the spinal cord of EAE diseased chimeras in which the CNS is NG2 deficient (wild-type  $\rightarrow$  knockout chimeras). Furthermore, our *in vitro* data demonstrate that NG2 expression can be induced in bone marrow-derived macrophages [CD11b<sup>+</sup> and F4/80<sup>+</sup> expression of these cells shown in (Moransard *et al.*, 2010)]. Concurrent with our data is the observation of NG2<sup>+</sup>/F4/80<sup>+</sup> macrophages outside the CNS (Tigges *et al.*, 2008). The quantitative appreciation of the NG2 expression by macrophages in EAE lesions is difficult due to the fact that the usually strongly CD11b<sup>+</sup> cuffs of the macrophages are 'flooded' by soluble NG2. Interestingly, our observations in the NG2 chimeras show that NG2<sup>+</sup>/CD11b<sup>+</sup> cells constitute a remarkably small part of all the CD11b<sup>+</sup> cells present in infiltrates in the CNS indicating that these cells form a distinct subpopulation. This small number of NG2<sup>+</sup>/CD11b<sup>+</sup> cells probably explains why these cells have evaded conclusive identification for so long.

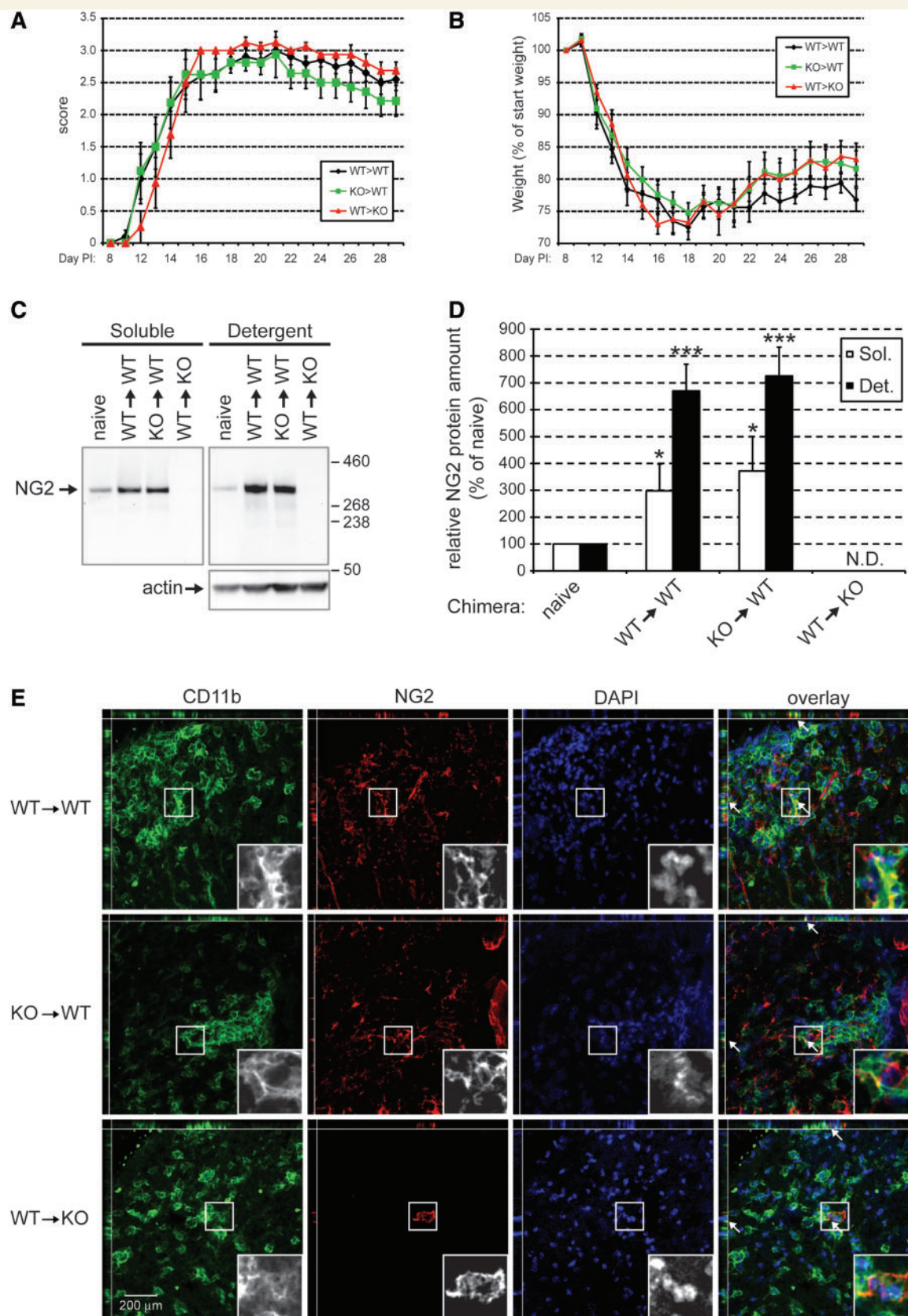
Notably, the studies mentioned above have used slightly different panels of antibodies and as a consequence it is not clear if we have identified the same cell population or if there are different subpopulations of NG2<sup>+</sup> macrophage-like cells. The NG2<sup>+</sup> cells we observe in the wild-type  $\rightarrow$  knockout chimeras are faintly CD11b<sup>+</sup> and a proportion of these cells also express the dendritic cell marker CD11c (data not shown), which would correspond well with the NG2<sup>+</sup>/OX42<sup>+</sup> cells identified by a number of groups (Bu *et al.*, 2001; Hampton *et al.*, 2004; Fiedorowicz *et al.*, 2008; Gao *et al.*, 2010; Wu *et al.*, 2010; Zhu *et al.*, 2010), which may be myeloid or inflammatory dendritic cells (Mildner *et al.*, 2009; Hesse *et al.*, 2010).

## NG2 plays no role in experimental autoimmune encephalomyelitis

Despite the fact that a number of reports have identified immune cells that express NG2 (Bu *et al.*, 2001; Jones *et al.*, 2002; Hampton *et al.*, 2004; Rezajooi *et al.*, 2004; Yokoyama *et al.*, 2006; Fiedorowicz *et al.*, 2008; Matsumoto *et al.*, 2008; Smirkin *et al.*, 2009; Gao *et al.*, 2010; Wu *et al.*, 2010; Zhu *et al.*, 2010)

### Figure 4 Continued

depicts areas evaluated for myelination. (Centre) magnified sections from first row (scale bars = 100  $\mu$ m). (Bottom) Electron micrographs of evaluated regions (scale bars = 1  $\mu$ m). (B–E) Percentage of myelinated axons compared to all visible axons (B), average axon diameter (C), average myelin thickness (D) and average G-Ratio (E) show no significant differences between wild-type and NG2 animals in either the Day 40 post-immunization or the control groups ( $P > 0.1$ ). Animals at Day 40 post-immunization show significantly reduced myelination compared to healthy control animals ( $*P < 0.05$ ;  $**P < 0.01$ ). In all the graphs, each data point represents an individual animal. Mean values are also depicted.



**Figure 5** CNS resident cells are responsible for increased NG2 expression and accumulation in and around areas of leucocyte infiltration. (A and B) EAE progression and weight loss of the different chimeras. Wild-type → wild-type  $n = 10$ , knockout → wild-type  $n = 8$ , wild-type → knockout  $n = 8$ . The fluctuations in weight correlate well with the degree of disability. EAE progression is comparable in all

(continued)

and increases in NG2 appear to be a pronounced injury response both in the CNS and PNS (Levine, 1994; Nishiyama *et al.*, 1997; Di Bello *et al.*, 1999; Moon and Fawcett, 2001; Jones *et al.*, 2002; Morgenstern *et al.*, 2002; Reynolds *et al.*, 2002; Tang *et al.*, 2003; Rezajooi *et al.*, 2004; Asher *et al.*, 2005; Tan *et al.*, 2005; Hossain-Ibrahim *et al.*, 2007), in depth studies investigating the role of NG2 in the immune response have been lacking. Only two recent studies have tried to address the role of NG2<sup>+</sup> macrophage/microglia-like cells in CNS inflammation directly. Data by Gao *et al.* (2010) indicate that NG2 mediates the induction of iNOS and inflammatory cytokine but not chemokine expression in lipopolysaccharide-activated microglia. A report by Smirkin *et al.* (2009) identifies several neuroprotective factors expressed by Iba1<sup>+</sup>/NG2<sup>+</sup> macrophage-like cells and suggests that these cells may ameliorate ischaemic brain damage. However, our data demonstrate that NG2 deficiency has no significant effect on EAE progression, leucocytes extravasation or migration within the CNS, demyelination or the extent of neuronal damage, indicating that NG2 does not play an important role in macrophage/microglia function in autoimmunity.

In addition, NG2 accumulation has no effect on remyelination. Although we did not address axon outgrowth directly, the comparably clinical recovery and extent of neuronal damage and de- and remyelination at remission in NG2 knockout mice and wild-type littermates indicate that NG2 is not a major inhibitory or stimulatory factor in axon outgrowth, which is in good agreement with the lack of improvement in axon outgrowth observed in the NG2 knockout mouse after spinal cord injury (de Castro *et al.*, 2005; Hossain-Ibrahim *et al.*, 2007).

In summary, the TGF- $\beta$ -induced expression of NG2 in macrophages *in vitro* and expression of NG2 by a small population of blood-borne macrophages in the CNS of EAE diseased mice are indicative of a novel, potentially anti-inflammatory, macrophage subpopulation. However, neither CNS-derived nor peripheral macrophage expressed NG2 plays a role in the course of CNS autoimmunity. Finally, although we have established here that secreted and accumulated NG2 is derived from CNS resident oligodendrocyte progenitor cells, the biological role of this rather prominent response of the CNS to injury remains an open question.

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## Supplementary material

Supplementary material is available at *Brain* online.

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### Figure 5 Continued

groups. (C and D) Western blot analysis and corresponding densitometric quantitation of NG2 in spinal cord of EAE diseased chimeras at remission. Soluble and detergent-extracted NG2 amounts are elevated in wild-type  $\rightarrow$  wild-type and knockout  $\rightarrow$  wild-type chimeras compared to naïve wild-type controls. NG2 is not detected in wild-type  $\rightarrow$  knockout spinal cord lysates. Increased cell-bound and secreted NG2 protein amounts are comparable between wild-type  $\rightarrow$  wild-type and K  $\rightarrow$  wild-type chimeras. (E) Immunohistochemistry for CD11b and NG2 on thoracic spinal cord cross-sections from EAE diseased chimeric mice at remission analysed by confocal microscopy. Wild-type  $\rightarrow$  wild-type chimeras show extensive NG2 accumulation in and around areas of leucocyte infiltration, which partly overlaps with CD11b immunoreactivity. Knockout  $\rightarrow$  wild-type mice show a similar degree of infiltration of CD11b<sup>+</sup> cells and a comparable degree of NG2 accumulation. The overlap between CD11b and NG2 staining appears less frequent. Wild-type  $\rightarrow$  knockout animals show a comparable infiltration of CD11b<sup>+</sup> cells. However, NG2 staining is almost completely absent. Only occasionally NG2<sup>+</sup> cells can be detected in the infiltrate (inserts). These cells also express low levels of CD11b (overlay and insert). Arrows indicate CD11b and NG2 co-localization. Inserts show electronically  $\times 2.35$ -magnified images of the boxed areas. Bars represent mean  $\pm$  SEM;  $n = 4$ ; \* $P < 0.05$ ; \*\*\* $P < 0.001$  versus control.



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